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Atty. Docket No. UCSF 048CIP  
UCSF Ref. No. SF97-042-2

Dated: 3/23/00

By: 

Mathew Otts

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In Re Application of:

German et al.

Serial No.: 09/254,988

Filing Date: June 11, 1999

Title: *Delivery of Therapeutic Gene Products by Intestinal Cell Expression*

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Group Art Unit: 1633

Examiner: D. Nguyen

Assistant Commissioner of Patents and Trademarks  
Washington, DC 20231

**DECLARATION OF STEPHEN S. ROTHMAN, Ph.D.**  
**UNDER 37 C.F.R. § 1.132**

Dear Sir:

1. I, Stephen Rothman, declare and say I am a resident of Berkeley, California. My residence address is 98 Acacia Avenue, Berkeley, California 94708. I am a co-inventor of the invention of the claims of the above-referenced patent application.

2. I hold a Bachelor degree in Zoology which I received from the University of Pennsylvania in 1956. I further hold a D.D.S. degree which I received from the University of Pennsylvania in 1961. I further hold a Ph.D. in Physiology which I received from the University of Pennsylvania in 1964. I am currently a Professor of Physiology at the University of California, San Francisco, Third and Parnassus Avenues, San Francisco, California 94143-0534. I am an expert in the fields of gene therapy, molecular biology, cell biology, cellular physiology and biophysics, and the anatomy and physiology of the gastrointestinal system.

3. I am an inventor of the claims of the above-identified patent application. I directed others and personally performed the research leading to the invention disclosed and claimed therein.

4. I have read the Office Action dated November 23, 1999 in this application and understand that the Examiner has rejected pending claims 1-10 and 14-17 on the basis that the specification allegedly does not provide an enabling disclosure for the full scope of the claims. Specifically, the Office Action stated that the specification does not provide enablement for routes of administration other than direct injection. The Office Action cited a number of journal articles as support for the contention that routes of administration other than direct injection are unpredictable.

#### OVERVIEW

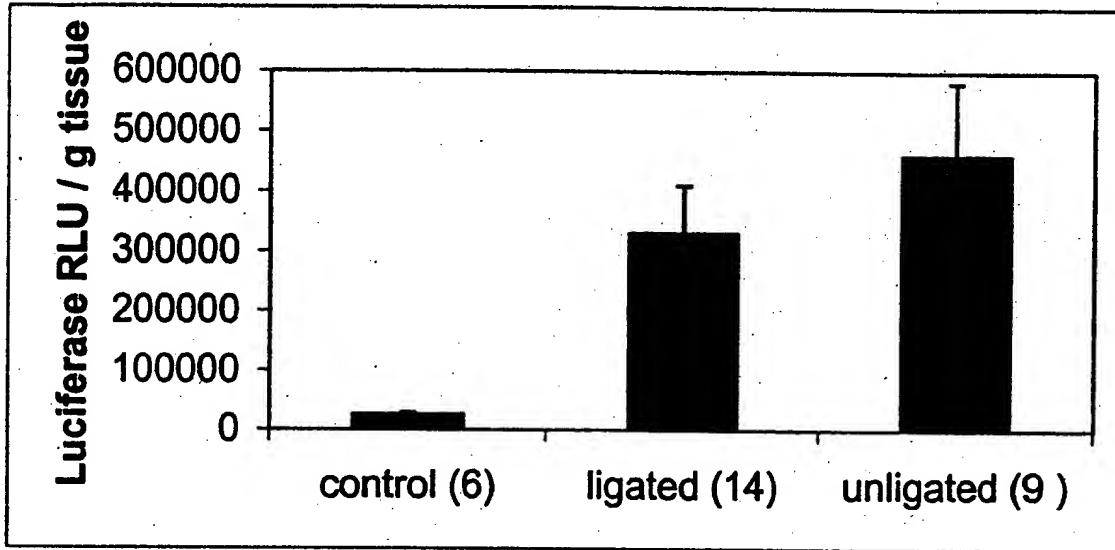
5. The experimental data presented in the following sections provide further evidence of the fact that routes of gastrointestinal tract administration other than direct injection can be accomplished by known, art-accepted methods. These methods include (1) introduction into the duodenum; and (2) oral administration. The results were the same, regardless whether the construct is introduced by oral administration, by introduction into the duodenum, or by direct injection. Regardless of the route of administration, an introduced DNA construct enters an intestinal epithelial cell and is expressed therein. Accordingly, the specification is indeed enabling for a method of introducing, by various routes of administration, including oral administration, a formulation comprising a DNA construct not packaged in a viral particle directly into a GI tract lumen of a mammalian subject, wherein the construct enters an intestinal epithelial cell.

## **I. DELIVERY OF A DNA CONSTRUCT TO INTESTINAL EPITHELIAL CELLS BY INTRODUCING THE CONSTRUCT INTO THE UPPER DUODENUM**

6. DNA constructs were introduced into an intestinal lumen of mammalian subjects via introduction into the upper duodenum, using the following protocol. Male BALB/c mice (specific pathogen free, Harlan Co., CA) weighing 17-20 g were used in this study. Animals were fasted overnight (water provided *ad libitum*). After anaesthetizing the animals with Isoflurane, a midline laparotomy was performed. The duodenum was exposed through the incision. At 2 cm below the pylorus, the lumen of a segment of duodenum 5 cm in length was temporarily isolated from the rest of the intestines by ligation orally and aborally by surgical ligatures. 100  $\mu$ l of deionized water containing 128  $\mu$ g of luciferase-encoding or, as a control, human growth hormone (hGH)-encoding plasmid DNA was introduced into the lumen over a period of 2-3 minutes. At 45 minutes after introduction of the construct into the lumen, the ligatures were removed, 0.1 ml of an ampicillin solution (15 mg/ml) was injected into the peritoneal cavity, and the abdomen closed with surgical sutures. In other experiments, the construct was introduced into the upper duodenum without prior isolation and ligation of the duodenum. Afterwards, the animals were allowed to recover. 24 or 48 hours after treatment, mice were euthanized, and the duodenum removed and rinsed with cold phosphate buffered saline. The duodenal tissue was then homogenized in cold lysis buffer (1.0 ml per 0.1 g tissue). Luciferase activity was then determined using a Monolight 2010 luminometer (Analytic Luminescence Laboratories) by measuring light emission from a 100  $\mu$ l aliquot of the lysis homogenate for a 10-second period. The amount of luciferase is expressed in terms of relative light units (RLU).

7. A DNA construct comprising a reporter gene encoding luciferase was introduced into the upper duodenum of mice, just aboral to the pyloric canal separating the stomach from the intestines, as described in paragraph 6, above. As shown in Figure 1, this route of administration produces reliable expression of the reporter gene in intestinal epithelial cells. 87% of the animals

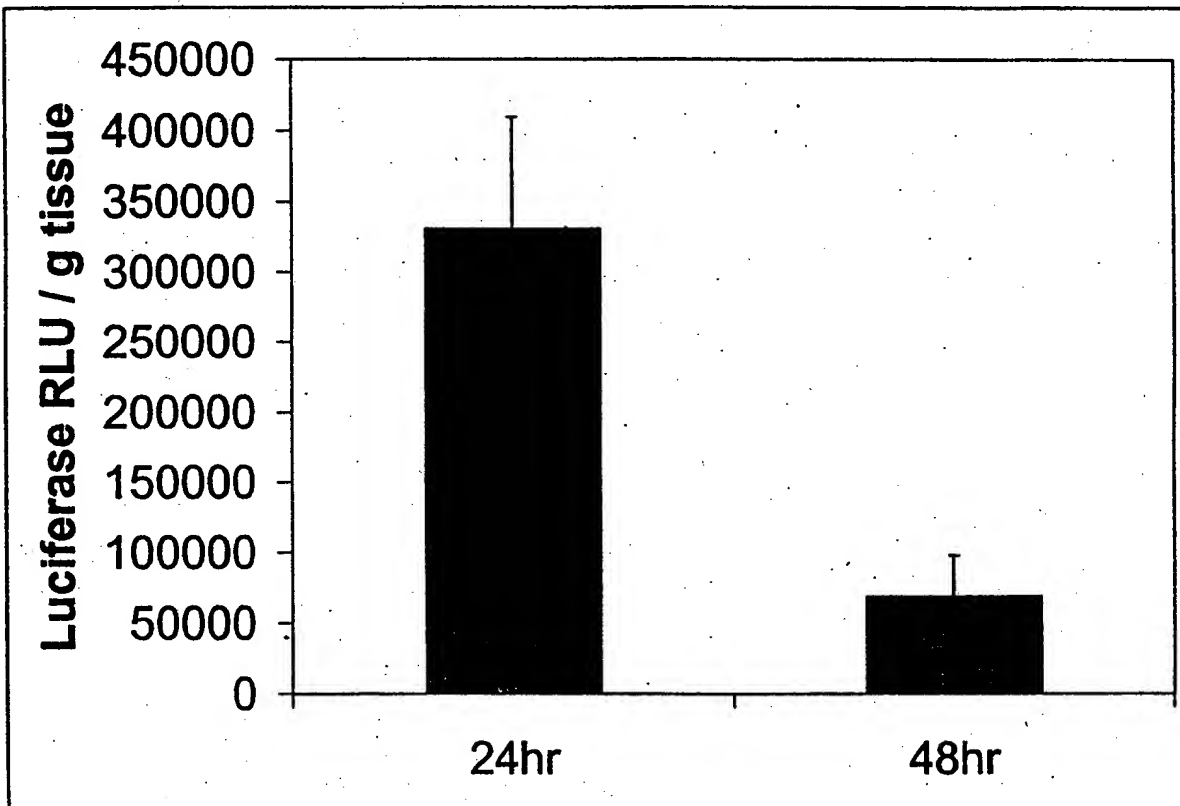
expressed the reporter gene in intestinal epithelial cells. On average, the values obtained were approximately 20 times greater than background levels of expression. High levels of expression were observed, regardless whether the duodenum was ligated or unligated.



**Figure 1.** Expression of luciferase in the mouse duodenum. 24 hours after introduction of the luciferase-encoding construct, luciferase activity was measured, and is expressed as relative light units. Values are shown for controls (a plasmid encoding hGH instead of luciferase), animals in which the construct was introduced into ligated segments of the proximal duodenum, and animals in which the construct was administered by introduction into unaltered duodenum, as occurs physiologically. Results are shown as the mean  $\pm$  SE. N for each group is in parentheses.

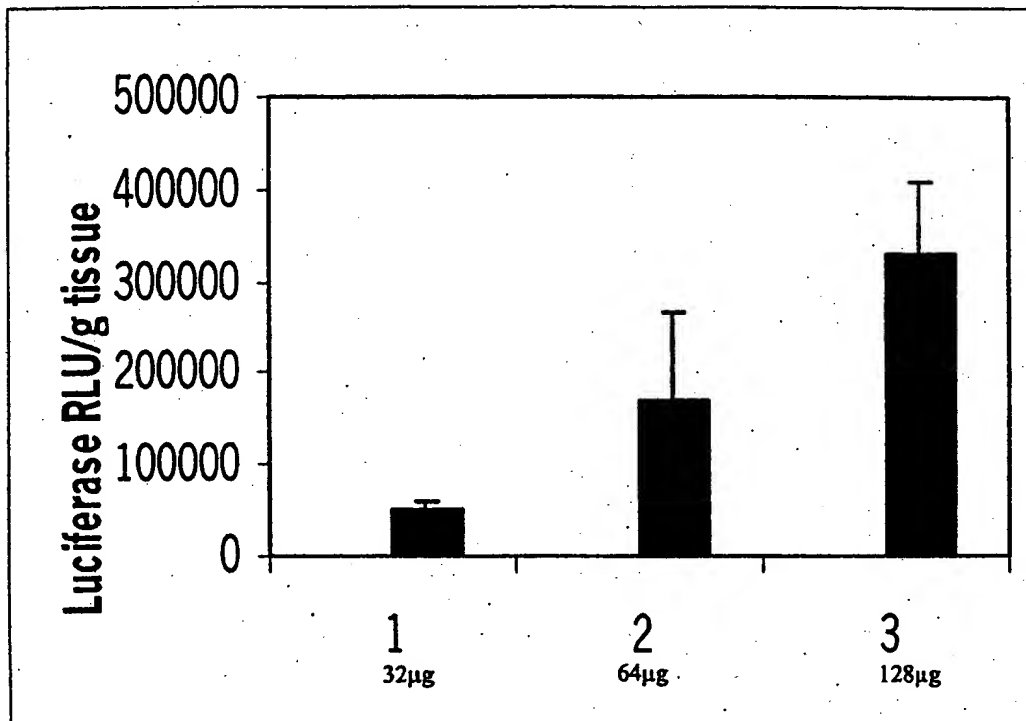
8. As shown in Figure 2, expression in intestinal cells was high 24 hours after administration of the DNA construct to the animals, and, after 48 hours, decreased to nearly background levels. These results demonstrate that expression occurred in the rapidly turning over cells of the surface epithelium of the intestine. These cells are primarily absorptive cells. Thus,

the site of expression is the rapidly turning over cells on the surface epithelium of the intestine, not the long-lasting crypt cells or stem cells.



**Figure 2.** Luciferase activity in the duodenum 48 hours after intraluminal administration of the luciferase-encoding construct. Expression of luciferase is reduced by 80% between 24 and 48 hours, suggesting that expression occurs in rapidly turning-over cells. Data are shown as the mean  $\pm$  SE. N is 14 for 24 hours and 4 for 48 hours.

9. As shown in Figure 3, luciferase expression from the luciferase-encoding DNA construct in the duodenum is dose-dependent. Animals were administered 32, 64, or 128  $\mu$ g luciferase-encoding DNA constructs introduced into the duodenum, as described above in paragraph 6. Data are shown as mean  $\pm$  SE for three separate experiments at each of the three plasmid doses.



**Figure 3.** Luciferase expression in the duodenum is dose-dependent. Data are shown as mean  $\pm$  SE for three separate experiments at each of three different plasmid doses (32  $\mu$ g, 64  $\mu$ g, and 128  $\mu$ g DNA).

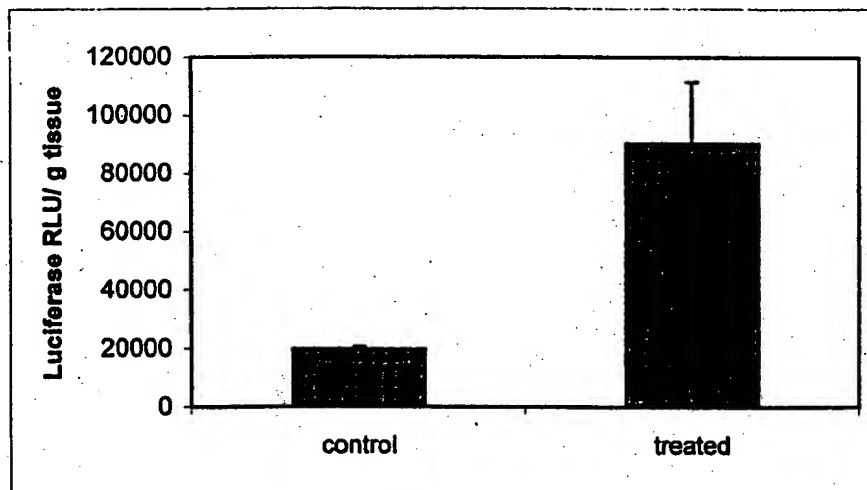
#### SUMMARY OF RESULTS

10. The results shown above demonstrate unequivocally that a DNA construct can be introduced into a GI tract lumen of a mammalian subject by an intraluminal route of delivery, resulting in expression of a protein encoded by the construct in intestinal epithelial cells. Introduction of a DNA construct into an intestinal lumen of a mammalian subject by this route is reliable. This is shown by the fact that (1) expression of a gene encoded by the construct was high; (2) expression of the encoded gene was dose-dependent; and (3) the results were reproducible. It is also important to note that expression was seen in the rapidly turning over epithelial cells, not in the crypt cells or stem cells, which cells are the focus of work by others.

## II. DELIVERY OF A DNA CONSTRUCT TO INTESTINAL EPITHELIAL CELLS BY INTRODUCING THE CONSTRUCT INTO A MAMMALIAN HOST VIA ORAL ADMINISTRATION

11. DNA constructs were delivered into an intestinal lumen of a mammalian subject via oral administration, using the following protocol. Male BALB/c mice weighing 17-20 grams were used in this study. Animals were fasted overnight. After lightly anesthetizing the animals with Isoflurane, a PE-20 polyethylene catheter was introduced through the mouth into the stomach. 0.1 ml of deionized water containing 128  $\mu$ g of luciferase-encoding plasmid DNA was administered by means of the catheter. The tubing was removed after administration was complete, and the animals were awake in 1-2 minutes. 24 hours after administration of the plasmid DNA, the mice were euthanized, the duodenum removed, and the tissue treated as described above for measuring luciferase activity levels. The control was a human growth hormone (hGH)-encoding plasmid DNA.

12. The results are shown in Figure 4.



**Figure 4.** Luciferase activity in the duodenum 24 hours after oral administration of plasmid DNA by gavage, as compared to a control in which the construct comprises a gene encoding hGH instead of luciferase.

### SUMMARY OF RESULTS

13. These results demonstrate unequivocally that a DNA construct can reproducibly be introduced into the GI tract lumen of a mammalian subject by oral administration, such that the construct enters an intestinal epithelial cell and is expressed therein. These results are the same as the results obtained when a DNA construct is introduced by into the GI tract lumen of a mammalian subject direct injection into an intestinal lumen.

14. Gavage is an art-accepted way to introduce a drug orally into experimental animals. Commonly, oral drugs are administered to rats by gavage, which involves instillation of the material directly into the stomach by means of a catheter introduced into the animal's mouth and then passed through the esophagus into the stomach. Materials delivered by gavage pass out of the stomach through the pyloric canal into the upper small intestine or duodenum. Gavage is used in experimental animals in lieu of oral administration, since gavage provides for more precise control of oral dose administered. Oral delivery into the mouth of experimental animals is less accurate due to problems associated with administration into the mouth. Thus, this method of introducing a DNA construct into the intestine is equivalent to oral delivery.

### CONCLUSION

15. The data presented above demonstrate the fact that the specification is indeed enabling for a method of introducing, by various routes of administration, including oral administration, a formulation comprising a DNA construct not packaged in a viral particle directly into a GI tract lumen of a mammalian subject, wherein the construct enters an epithelial cell. Data are provided showing that the results obtained were the same, regardless whether the construct was introduced by oral administration, or by introduction into the duodenum. These results are comparable to results obtained by direct injection into an intestinal lumen.

16. I heroby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code, and that such will false statements may jeopardize the validity of the application or any patent issuing thereon.

Date 3/22/00

Stephen S. Rothman

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